HCV Vaccines

The present invention relates to HCV vaccines.

The immune system is a complex network of inter-related cell types and molecules, which has evolved in order to protect multicellular organisms from infectious microorganisms. It can be divided into the evolutionary older innate (or natural) immunity and adaptive (or acquired) immunity. The innate immune system recognises patterns, which are usually common and essential for pathogens. For this limited number of molecular structures germline encoded receptors have evolved. By contrast, cells of the adaptive immune system – B and T lymphocytes – can recognise a huge variety of antigenic structures. The receptors, termed according to the cell types expressing them, B cell receptor (BCR, its soluble versions are called antibodies) and T cell receptor (TCR, only cell-surface associated forms) are generated by somatic recombination and show a clonal distribution. Thus, initially there is only small number of cells with a certain specificity. Upon antigen encounter these cells start to divide (clonal expansion) to generate an effector population able to cope with the antigen. After elimination of antigen a specialised sub-population of cells specifically recognising this antigen remains as immunological memory. Taken together the adaptive immune system is slow (compared to innate immunity), however specific and it improves upon repeated exposure to a given pathogen/antigen.

T cells have a central role in adaptive immunity. Their receptors (TCRs) recognise "major histocompatibility complex" (MHC or HLA):peptide complexes on the surface of cells. These peptides are called T cell epitopes and represent degradation products of antigens. There are two major classes of T cells: CD8-positive cytotoxic T cells (CTL) are restricted to MHC class I. CD4-positive helper T cells (HTL) are restricted to MHC class II. HTL are essential for many features of adaptive immunity: activation of so called "professional antigen-presenting cells" (APCs), immunoglobulin (Ig) class switch, the germinal center reaction and Ig affinity maturation, activation of CTL, immunological memory, regulation of the immune response and others.

MHC molecules collect peptides inside the cell and present them
on the cell surface to TCRs of T cells. There are two major
classes of MHC, class I recognised by CD8-positive CTL and class
II recognised by CD4-positive HTL.

MHC class I molecules consist of a membrane-anchored alpha-chain
of 45 kDa and the non-covalently attached b2-microglobulin (b2m)
of 12 kDA. Resolution of the 3-dimensional structure by X-ray
crystallography (Stern and Wiley 1994) revealed that the alpha-
chain possesses a cleft, which is closed at both ends and accom-
modates peptides from 8 to 11 amino acids length. Class I mo-
lecules are ubiquitously expressed, and the peptides they
present originate from cytoplasmic proteins. These are degraded
by the proteasome, and the resulting peptides are actively
transported into the endoplasmatic reticulum (ER). There, with
the help of several chaperones, MHC:peptide complexes are formed
and transported to the cell surface (Heemels 1995). Thus, MHC
class I mirrors the proteome of a cell on its surface and allows
T cells to recognise intracellular pathogens or malignant cells.

MHC class II molecules consist of two membrane-anchored proteins
(alpha- and beta-chain) of 35 kDa and 30 kDa, respectively.
These together form a cleft, open at both ends, which can accom-
modate peptides of variable length, usually from 12 to 25 amino
acids. Despite these differences, class I and II molecules share
surprising structural similarity (Stern and Wiley 1994). Class
II molecules are only expressed on professional APC including
dendritic cells (DC), B-cells and macrophages/monocytes. These
cells are specialised in taking up and processing antigens in
the endosomal pathway. Immediately after their biosynthesis,
class II molecules are complexed by the so-called invariant
chain (Ii), which prevents binding of peptides in the ER. When
vesicles containing class II:Ii complexes fuse with endosomes
containing degradation products of exogenous antigen, Ii is de-
graded until the MHC binding cleft is only complexed by the so-
called CLIP peptide. The latter is with the help of chaperones
like HLA-DM exchanged by antigenic peptides (Villadangos 2000).
Finally, MHC:peptide complexes are again presented on the sur-
face of APCs, which interact in numerous ways with HTL.

Being both polygenic and extremely polymorphic, the MHC system
is highly complex. For the class I alpha-chain in humans there
are three gene loci termed HLA-A, -B and -C. Likewise, there are
three class II alpha-chain loci (DRA, DQA, DPA); for class II
beta-chain loci the situation is even more complex as there are
four different DR beta-chains (DRB1,2,3,5) plus DQB and DPB.
Except the monomorphic DR alpha-chain DRA, each gene locus is
present in many different alleles (dozens to hundreds) in the
population (Klein 1986). Different alleles have largely distinct
binding specificities for peptides. Alleles are designated, for
example, HLA-A*0201 or HLA-DRB1*0401 or HLA-DPA*0101/DPB*0401.

T cell epitopes have been identified by a variety of approaches
(Van den Eynde 1997). T cell lines and clones have for instance
been used to screen cDNA expression libraries for instance in
the context of COS cells transfected with the appropriate HLA-
molecule. Alternatively, biochemical approaches have been pur-
sued. The latter involved elution of natural ligands from MHC
molecules on the surface of target cells, the separation of
these peptides by several chromatography steps, analysis of
their reactivity with lymphocytes in epitope reconstitution as-
says and sequencing by mass spectrometry (Wölfel et al. 1994,
Cox et al. 1994).

Recently the advent of highly sensitive cytokine detection as-
says like the IFN-gamma ELIspot allowed using lymphocytes dir-
ectly ex vivo for screening of overlapping synthetic peptides
(Maecker 2001, Kern 2000, Tobery 2001). Primarily, Kern et al.
(1999&2000) used arrays of pools of overlapping 9mer peptides to
map CD8+ T cell epitopes in vitro. Later, Tobery et al., 2001
modified this approach and demonstrated that pools containing as
many as 64 20mer peptides may be used to screen for both CD8+
and CD4+ T cell epitopes in mice. Both these methods were based
on the monitoring of antigen-specific response by measuring IFN-
gamma production either by intracellular staining (Kern et al
2000) or in ELIspot assay (Tobery et al., 2001). By use of mix-
tures of 15-mers the CD4+ T cell responses are approximately
equal to those detected when whole soluble protein was used as
an antigen, while -not surprising- the CD8+ T cell responses are

significantly higher than the often negligible responses detected with soluble protein stimulation. Furthermore, the CD8+ T cell responses to a mixture of 15 amino acid peptides are similar to those obtained with a mix of 8-12 amino acid peptides, selected to represent known MHC class I minimal epitopes. Most probably peptidases associated with the cell membrane are responsible for "clipping" peptides to optimal length under these circumstances (Maecker et al, 2001).

An interesting alternative is to screen synthetic combinatorial peptide libraries with specific lymphocytes. For instance, a decapeptide library consisting of 200 mixtures arranged in a positional scanning format, has been successfully used for identification of peptide ligands that stimulate clonotypic populations of T cells (Wilson, et al., J. Immunol., 1999, 163:6424-6434).

Many T cell epitopes have been identified by so called "Reverse immunological approaches" Rammensee 1999). In this case the protein giving rise to a potential T cell epitope is known, and its primary sequence is scanned for HLA binding motifs. Typically dozens to hundreds of candidate peptides or even a full set of overlapping peptides are synthesised and tested for binding to HLA molecules. Usually, the best binders are selected for further characterisation with regard to their reactivity with T cells. This can for instance be done by priming T cells in vitro or in vivo with the help of HLA transgenic mice.

Hepatitis C Virus (HCV) is a member of the flaviviridiae chronically infecting about 170 million people worldwide. There are at least 6 HCV genotypes and more than 50 subtypes have been described. In America, Europe and Japan genotypes 1, 2 and 3 are most common. The geographic distribution of HCV genotypes varies greatly with genotype 1a being predominant in the USA and parts of Western Europe, whereas 1b predominates in Southern and Central Europe (Bellentani 2000).

HCV is transmitted through the parenteral or percutan route, and replicates in hepatocytes. About 15% of patients experience acute self-limited hepatitis associated with viral clearance and

recovery. About 80% of infected persons become chronic carriers. Infection often persists asymptomatically with slow progression for years, however ultimately HCV is a major cause of cirrhosis, end-stage liver disease and liver cancer (Liang 2000). Strength and quality of both HTL and CTL responses determine whether patients recover (spontaneously or as a consequence of therapy) or develop chronic infection (Liang 2000).

Standard therapy of HCV comprises a combination of pegylated interferon-alpha and the antiviral ribavirin. Virologic responses are, depending on the genotype, achieved in about 50% of HCV patients. The low tolerability and the considerable side effects of this therapy clearly necessitate novel therapeutic intervention including therapeutic vaccines (Cornberg 2002). However, presently the detailed understanding of which epitopes in which MHC combination lead to successful immune responses is lacking (Ward 2002). Therefore, a comprehensive analysis of the T-cell response against the entire HCV is required for development of therapeutic epitope-based vaccines.

The HCV virion contains a 9.5-kilobase positive single-strand RNA genome encoding a large single polyprotein of about 3000 amino acids. The latter is processed to at least 10 proteins by both host and HCV-enoded proteolytic activities (Liang 2000). Importantly, the HCV RNA-dependent RNA polymerase is error prone giving rise to the evolution of viral quasispecies and contributing to immune-escape variants (Farci 2000).

Although many proposals have been made in the art in the last 15 years and many promising antigen candidates have been proposed during this time, there is still no HCV vaccine on the market (nor a satisfactory therapeutic treatment; the only available treatment, as mentioned above a combination of alpha interferon and ribavirin, is efficacious only in a minority of patients). Early after the publication of the HCV sequences, a myriad of epitopes and antigens were suggested as effective means for combatting HCV disease (e.g.: Koziel et al., J. Virol. 67(12), 7522-7532; Shirai et al., J. Virol. 68(5) (1994), 3334-3342; Leroux-Roels et al., Hepatology 23(1) (1996), 8-16; Hoffmann et al., Hepatology 21(3) (1995), 632-638; Sarobe et al., J. Clin. Invest.

102(6)(1998), 1239-1248; Battegay et al., J. Virol. 69(4)(1995),
2462-2470; Wentworth et al., Int. Imunol. 8(5)(1996), 651-659;
Rehermann et al., J. Virol. 70(10)(1996), 7092-7102; Diepolder
et al., J. Virol. 71(8)(1997), 6011-6019; Lamonaca et al., Hep-
atology 30(4)(1999), 1088-1098; WO00/11186, WO95/12766,
WO95/27733, WO94/20127, WO95/25122, WO95/22317, WO94/25601,
WO92/03458 and WO93/00365). Additionally, important proof-of-
principle ex vivo neutralisation studies have been undertaken
with chimpanzees, the other species other than homo sapiens that
is susceptible to chronic HCV infection. Indeed, the study of
immune responses induced in individuals and chimpanzees with
acute resolved versus chronic infections has so far revealed
only an emerging picture of potential protective immune re-
sponses following the establishment of active viral infection.
However, the correlates of prophylactic vaccine protection fol-
lowing parenteral immunisation are expected to be largely extra-
hepathic prior to HCV exposure. Only a limited number of vaccine
efficacy studies have been performed with chimpanzees and these
studies showed - at best - a protection of 5 out of 7 animals
from infection after a low-dose homologous challenge with ex-
actly the same clone being used for immunisation and challenge.
In addition, HCV presents a high degree of variability which
provides HCV with the capacity to escape vaccine- or infection-
induced immune responses. In consideration of this problem, also
a combination of different large protein fragments as a DNA vac-
cine has been suggested as alternative vaccination strategy
(Rollier et al., J. Virol. 78(1)(2004), 187-196; Dunas-Carrera
et al., Biotech. Appl. Biochem. Imm. Publ. (29.September 2003;
BA20030112)).

Despite all the progress that has been made in the last 15, es-
pecially in the last 10 years there is still a pressing need to
develop new therapies and vaccination strategies for HCV (Heile
et al., J. Virol. 74(15)(2000), 6885-6892).

It is therefore an object of the present invention to provide
such effective therapies and vaccination strategies for HCV.

The present invention therefore provides a Hepatitis C virus
(HCV) vaccine comprising at least two epitopes, each from a dif-

ferent hotspot epitope, wherein a hotspot epitope is defined as
an epitope containing peptide selected from the group consisting
of
KFPGGGQIVGGVYLLPRRGPRLGVRATRK,

GYKVLVLNPSVAAT,

AYAAQGYKVLVLNPSVAAT,

DLMGYIP(A/L)VGAPL,

GEVQVVSTATQSFLATCINGVCWTV,

HMWNFISGIQYLAGLSTLPGNPA,

VDYPYRLWHYPCT(V/I)N(F/Y)TIFK(V/I)RMYVGGVEHRL,

AAWYELTPAETTVRLR,

GQGWRLLAPITAYSQQTRGLLGCIV,

IGLGKVLVDILAGYGAGVAGALVAFK,

FTDNSSPPAVPQTFQV,

LEDRDRSELSPLLLSTTEW,

YLVAYQATVCARAQAPPPSWD,

MSTNPKPQRKTKRNTNR,

LINTNGSWHINRTALNCNDSL,

TTILGIGTVLDQAET,

FDS(S/V)VLCECYDAG(A/C)AWYE,

ARLIVFPDLGVRVCEKMALY,

AFCSAMYVGDLCGSV,

GVLFGLAYFSMVGNW,

VVCCSMSYTWTGALITPC,

TRVPYFVRAQGLIRA and

TTLLFNILGGWVAAQ.

In the application WO04/024182, filed 27 August 2003 (claiming a
priority A 1367/2002 of 13 September 2002), a novel method for
isolating HCV peptides useful in vaccination, especially HCV T
cell epitopes, is described. In this application the concept of
"HCV hotspot epitopes" was disclosed for the first time: a T-
cell epitope "hotspot" is defined as a short peptide sequence at
least comprising more than one T-cell epitope. For example, two
or more epitopes may be located shortly after each other
(shortly being defined as less than 5-10 amino acids), or dir-
ectly after each other, or partially or even fully over-lapping.
Hotspots may contain only class I or class II epitopes, or a
combination of both. Epitopes in hotspots may have different HLA
restrictions.

Due to the highly complex and selective pathways of class I and class II antigen processing, referred to in the introduction, T-cell epitopes cannot be easily predicted within the sequence of a polypeptide. Though widely used, computer algorithms for T-cell epitope prediction have a high rate of both false-negatives and false-positives.

Thus, as even individual T-cell epitopes are not obvious within the sequence of a polypeptide, the same is even more the case for hotspots. Several radically different experimental approaches are combined according to the present invention for T-cell epitope identification, including epitope capture, HLA-transgenic animals and in vitro stimulation of human mononuclear cells. All three approaches are systematically applied on overlapping peptides spanning the antigen of interest, enabling comprehensive identification of epitopes. Upon such a comprehensive analysis, not limited to a particular HLA allele, but rather unravelling all possibly targeted epitopes within a population, epitope hotspots may become apparent. Within an antigen, only few if any sequences show characteristics of hotspots. Thus the identification of a hotspot is always a surprising event.

T-cell epitope hotspots offer important advantages: Hotspots can activate and can be recognised by different T-cell clones of a subject. Hotspots (when comprising epitopes with different HLA restriction) can interact with T-cells from different non HLA-matched individuals.

Epitope-based vaccines, so far have aimed at selected prevalent HLA-alleles, for instance HLA-A2, which is expressed in about half of Caucasians. Since other alleles are less frequent, epitope-based vaccines with broad worldwide population coverage will have to comprise many different epitopes. The number of chemical entities (for instance peptides) of a vaccine is limited by constraints originating from manufacturing, formulation and product stability.

Hotspots enable such epitope-based vaccines with broad worldwide population coverage, as they provide a potentially high number

of epitopes by a limited number of peptides. Indeed, the prior art (see the aforementioned references concerning the epitopes discussed in the art and the fact that a HCV vaccine has not been developed yet) as it currently stands has clearly shown that the development of HCV vaccines has been regarded as problematic and that there has been a general failure to develop HCV vaccines and therapies despite the abundance of references through the last 10 years teaching the efficacy of HCV antigens in eliciting humoral and cellular immune response in several infection models. The treatment and prevention of HCV infection is wrought with complexity and severe unpredictability.

The concept of the present invention by providing HCV vaccines based on the HCV hotspot epitope characterisation now enables for the first time a HCV vaccination system enabling both, a suitable and specific prophylactic vaccination and an efficient treatment regimen for HCV infected patients, especially chronically infected patients.

It could be shown by the inventors of the present application that the use of only one or two specific epitopes in the HCV vaccine is not sufficient for satisfactory results in vaccination. Also the concept of providing a mixture of HCV proteins (Core, E1 and E2 as a DNA vaccine; Duenas-Carrera et al. (2003)) or a mixture of large fragments of such HCV proteins (Core, E1, E2 and NS3 as DNA vaccines; Rollier et al., (2004)) did not bring the desired results.

The HCV vaccines according to the present invention contain short polypeptides containing epitopes from the hotspot epitopes as listed above. The present vaccines are not dependent on the risks and uncertainties with respect to DNA vaccines (lack of long lasting immune response; largely extrahepatic action; mode of action, localisation, etc.). Moreover the present invention also does not face the severe manufacturing problems with the production of full proteins or long-chained protein fragments.

The hotspot epitopes disclosed herein have been identified via functional assays using primarily a Th1/Tc1-type reaction (i.e. interferon gamma) as read-out. It is well known that (whole or

at least large fragment) antigens do not only contain such agonist epitopes, but may also encode antagonist ligands. Such antagonists potentially present in whole antigens, but absent in hotspots may impede immunogenicity and efficacy of the vaccine. Moreover, whole antigens will almost certainly induce a humoral immune response. Such an antibody response has per se little effect on HCV residing within cells, but it can severely compromise the desired T-cell response. The epitopes being derived from hotspots according to the present invention, due to their restricted length, have a much lower probability to induce unwanted antibody responses. Finally, only relatively small parts of HCV antigens are conserved between different genotypes. Thus, whole antigens used in a vaccine may induce responses against non-conserved regions or epitopes, irrelevant for most of the patients. In contrast, the hotspots disclosed herein are derived from regions conserved > 80% among the major HCV genotypes 1, 2 and 3.

HLA-super types (as disclosed in e.g. WO 01/21189) describe the fact that in some cases a given HLA-class I T-cell epitope is not exclusively restricted to on and only one HLA-allele but can also be recognised by T-cells in context of another HLA-allele (example: a peptide may bind to HLA-B*0702 and -B*3501). Such peptides by nature have broader HLA coverage in the population and are therefore interesting vaccine candidates.

In contrast a T-cell epitope hotspot according to the present invention significantly differs from such a HLA-super type, because a hotspot according to the present invention is a peptide region within a protein that shows an unusual high number of different adjacent and even overlapping epitopes. Hotspot deliver a high number of epitopes in relatively short regions, at the same time the proper processing of the epitopes within hotspots is guaranteed.

Indeed, the hotspots according to the present invention show a remarkably low variation between different HCV species which makes them even more favourable for vaccine approaches, because also heterologous challenge is possible even though only small polypeptides are used. In any way, the epitopes provided with

the present invention do not exceed a length of 100 amino acids, but are in general significantly shorter. Preferably the length of the epitopes according to the present invention is at least 6, 7, 8, 9, 10, 11 or 12 amino acids. Maximum lenghts of 13, 15, 20, 25, 30, 40 or 50 amino acids are also regarded as preferred.

According to the present invention the epitopes for a given vaccine may be selected from the hotspot regions as defined above. Of course, either the whole hotspot region may be provided in the vaccine or only parts thereof (as long as the part contains at least one epitope). It is, however advantageous, if one or more (e.g. 2, 3, 4, 5 or 6) epitopes are provided in the vaccine which contain the whole hotspot region or at least a region containing more than one epitope. The epitopes to be used according to the present invention may also be sequence variants having deletions (of preferably 1, 2, 3, 4, 5, 6 or 7 amino acids) in the hotspot "master" sequence as defined above. Preferably, the deletions inside the hotspot sequence do not alter the epitopes; of course at least one epitope in the hotspot region has to be preserved despite the deletion.

It is specifically preferred to provide a mixture of epitopes in the vaccine with one group of peptides having only one epitope and the other group having more than one epitope. For example a vaccine which contains at least one or at least two peptides with one epitope and at least one or at least two peptides with more than one epitope (e.g. the whole hotspot sequence) is preferred.

The epitopes may be provided as polypeptides without any modifications; modifying the polypeptide chain with certain chemical or biological moieties may be appropriate, especially with respect to solubilising or pharmaceutical formulation considerations (e.g. as described in WO01/78767). The epitopes used in the present HCV vaccine may either comprise the whole hotspot region or a contiguous fragment thereof (containing at least one of the epitopes contained in the hotspot) or comprise a non-contiguous part of the hotspot (however, also containing at least one of the epitopes contained in the hotspot; in such a case one or more linkers (e.g. amino acid or peptide linkers) can be

provided between the non-contiguous parts of this hotspot).

According to a preferred embodiment, the HCV vaccine according
to the present invention comprises at least three, especially at
least four epitopes, each from a different hotspot epitope.

Even more preferred, the HCV vaccine is comprising at least five
epitopes, each from a different hotspot epitope. Although the
higher the number of epitopes is in such an epitope-based vac-
cin, the larger the problems with respect to formulation are, a
number of 4, 5 or 6 epitopes is the optimal balance between
broad range of protection capacities and formulation drawbacks,
especially with respect to production on an industrial scale.

In certain cases therefore also a HCV vaccine comprising at
least six epitopes, each from a different hotspot epitope may be
a preferred embodiment of the present invention. The optimal
number of hotspot epitopes is also dependent on the relative in-
teraction of the selected epitopes with each other which has
proven to be the major obstacle for certain combinations of
short lenght polypeptides in a vaccine.

Preferred HCV vaccines according to the present invention are
characterised in that the epitopes are selected from the follow-
ing groups:
KFPGGGQIVGGVYLLPRRGPRLGVRATRK, KFPGGGQIVGGVYLLPRRGPRL,
YLLPRRGPRL, LPRRGPRL, GPRLGVRAT, RLGVRATRK;
GYKVLVLNPSVAAT, AYAAQGYKVL, AYAAQGYKVLVLNPSVAAT;
DLMGYIPAV, GYIPLVGAPL, DLMGYIPLVGAPL;
CINGVCWTV, GEVQVVSTATQSFLAT, GEVQVVSTATQSFLATCINGVCWTV;
HMWNFISGIQYLAGLSTLPGNPA, MWNFISGIQYLAGLSTLPGN,   NFIS-
GIQYLAGLSTLPGNPA, QYLAGLSTL, HMWNFISGI;
VDYPYRLWHYPCTVNFTIFKVRMYVGGVEHRL, DYPYRLWHYPCTVNFTIFKI,
DYPYRLWHYPCTVNFTIFKV, VDYPYRLWHYPCTVNYTIFKIRMYVGGVEHRL,
DYPYRLWHYPCTVNYTIFKI, DYPYRLWHY, TVNYTIFKI, TINYTIFK,
TVNFTIFKV, HYPCTVNYTI, HYPCTVNFTI, RMYVGGVEHR;
AAWYELTPAETTVRLR, TPAETTVRL;
GWRLLAPITAYSQQTRGLLGCIV, TAYSQQTRGLLGCIV, TAYSQQTRGLLG, GQGWRL-
LAPITAYSQ, RLLAPITAY, GQGWRLLAPITAYSQQTRGLLGCIV, GQGWRLLAP-
ITAYSQQTRGLLG, AYSQQTRGLL, AYSQQTRGL; IGLGKVLVDILAGYGAGVAGAL-

VAFK, ILAGYGAGV, VAGALVAFK,   GYGAGVAGAL;

VVCCSMSYTWTGALITPC, SMSYTWTGALITP, SMSYTWTGAL, SYTWTGALI;

FTDNSSPPAVPQTFQV;

LEDRDRSELSPLLLSTTEW, LEDRDRSELSPLLLST, RSELSPLLL, ELSPLLLST,

DRDRSELSPL, LEDRDRSEL, LEDRDRSEL;

YLVAYQATVCARAQAPPPSWD, YLVAYQATV;

MSTNPKPQRKTKRNTNR, PQRKTKRNTNR, QRKTKRNTN, RKTKRNTNR, MSTNPKPQR,

MSTNPKPQK;

LINTNGSWHINRTALNCNDSL, NGSWHINRTALNCNDSL, LINTNGSWHI, RTALNCND-

SL, LINTNGSWHINRTALN, SWHINRTALN;

TTILGIGTVLDQAET, TTILGIGTV, TILGIGTVL;

FDSSVLCECYDAGAAWYE, FDSSVLCECYDAGCA, VLCECYDAGA,   VVLCECY-

DAGAAWYE;

ARLIVFPDLGVRVCEKMALY, ARLIVFPDL, RLIVFPDLGV, RVCEKMALY,

AFCSAMYVGDLCGSV;

GVLFGLAYFSMVGNW;

TRVPYFVRAQGLIRA;

TTLLFNILGGWVAAQ, LLFNILGGWV.

Generally, from one group of epitopes only one epitope should be
selected in order to achieve good results. Of course not all of
the hotspot epitopes listed above are equally powerful and ef-
ficacy may vary between different population groups. Preferably,
an individual vaccination strategy may be applied for each group
which is mainly based on HLA coverage. Therefore, a preferred
HCV vaccine according to the present invention is characterised
in that it comprises at least one epitope from at least two,
preferably at least three, even more preferred at least four of
the following hotspot epitopes:

KFPGGGQIVGGVYLLPRRGPRLGVRATRK,

AYAAQGYKVLVLNPSVAAT,

DLMGYIP (A/L) VGAPL,

GEVQVVSTATQSFLATCINGVCWTV and

HMWNFISGIQYLAGLSTLPGNPA.

For different specificites the HCV vaccine according to the
present invention comprises at least one epitope from at least
two, preferably at least three, especially at least four of the
following hotspot epitopes:

VDYPYRLWHYPCT (V/I) N (F/Y) TIFK (V/I) RMYVGGVEHRL,

AAWYELTPAETTVRLR,
GQGWRLLAPITAYSQQTRGLLGCIV,
IGLGKVLVDILAGYGAGVAGALVAFK,
FTDNSSPPAVPQTFQV,
LEDRDRSELSPLLLSTTEW,
YLVAYQATVCARAQAPPPSWD,
MSTNPKPQRKTKRNTNR and
LINTNGSWHINRTALNCNDSL.

For further different specificites the HCV vaccine according to
the present invention comprises at least one epitope from at
least two, preferably at least three, especially at least four
of the following hotspot epitopes:
TTILGIGTVLDQAET,
FDS(S/V)VLCECYDAG(A/C)AWYE,
ARLIVFPDLGVRVCEKMALY,
AFCSAMYVGDLCGSV,
GVLFGLAYFSMVGNW,
TRVPYFVRAQGLIRA and
TTLLFNILGGWVAAQ.

As an alternative to the precise antigens and epitopes listed
herein, antigen/epitope variants may be used in the vaccines ac-
cording to the present invention. Preferred variants are de-
signed according to homologues (containing one or more exchanges
being present in homologous HCV strains) or heteroclictic epi-
topes. Among preferred variants are those that vary from a se-
quence disclosed herein by conservative amino acid substitu-
tions. Such substitutions are those that substitute a given
amino acid in a polypeptide by another amino acid of like char-
acteristics. Typically seen as conservative substitutions are
the replacements, one for another, among the aliphatic amino
acids Ala, Val, Leu and Ile; interchange of the hydroxyl
residues Ser and Thr, exchange of the acidic residues Asp and
Glu, substitution between the amide residues Asn and Gln, ex-
change of the basic residues Lys and Arg and replacements among
the aromatic residues Phe and Tyr.

Further particularly preferred in this regard are variants hav-
ing the amino acid sequence of any polypeptide disclosed herein,

in which 1, 2, 3, 4 or 5 amino acid residues are substituted,
deleted or added, in any combination. Preferred among these are
silent substitutions, additions and deletions, which do not al-
ter the properties and activities of the polypeptide of the
present invention. Especially preferred in this regard are con-
servative substitutions. Specifically suitable amino acid sub-
stitutions are those which are contained in homologues for the
sequences disclosed herein. A suitable sequence variant of an
antigen or epitope as disclosed herein therefore includes one or
more variations being present in one or more strains or sero-
types of HCV (preferably 1, 2, 3, 4 or 5 amino acid exchanges
which are based on such homolog variations). Such antigens com-
prise sequences which may be naturally occurring sequences or
newly created artificial sequences. These preferred antigen
variants are based on such naturally occurring sequence vari-
ations, e.g. forming a "mixed sequence" for the antigenic re-
gions of the polypeptides according to the present invention.
For example, a given HCV sequence as disclosed herein may be
amended by including such one or more variations thereby creat-
ing an artificial (i.e. non-naturally occurring) variant of this
given (naturally occurring) antigen or epitope sequence.

It is clear that also epitopes or peptides derived from the
present epitopes or peptides by amino acid exchanges improving,
conserving or at least not significantly impeding the T cell ac-
tivating capability of the epitopes are covered by the epitopes
or peptides according to the present invention. Therefore, the
present epitopes or peptides also cover epitopes or peptides,
which do not contain the original sequence as derived from a
specific strain of HCV, but trigger the same or preferably an
improved T cell response. These epitopes are referred to as
"heteroclitic". These include any epitope, which can trigger the
same T cells as the original epitope and has preferably a more
potent activation capacity of T cells preferably in vivo or also
in vitro. Also the respective homologous epitopes from other
strains of HCV are encompassed by the present invention.

Heteroclitic epitopes can be obtained by rational design i.e.
taking into account the contribution of individual residues to
binding to MHC/HLA as for instance described by Ramensee et al.

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1999 (Immunogenetics 50: 213-219) or Sturniolo et al. 1999
(Nature Biotechnology 17: 555-562), combined with a systematic
exchange of residues potentially interacting with the TCR and
testing the resulting sequences with T cells directed against
the original epitope. Such a design is possible for a skilled
man in the art without much experimentation.

Another possibility includes the screening of peptide libraries
with T cells directed against the original epitope. A preferred
way is the positional scanning of synthetic peptide libraries.
Such approaches have been described in detail for instance by
Blake et al 1996 () and Hemmer et al. 1999 () and the references
given therein.

As an alternative to epitopes represented by the cognate HCV de-
rived amino acid sequence or heteroclitic epitopes, also sub-
stances mimicking these epitopes e.g. "peptidemimetica" or
"retro-inverso-peptides" can be applied.

Further chemical groups, especially amino acid residues, linkers
(and binding to carriers), transport facilitating groups, etc.,
may be added to the N- and/or C-terminus of the eptiopes in the
HCV vaccine according to the present invention. Amino acid at-
tachments as described in WO01/78767 are preferred.

Another aspect of the design of improved hotspot epitopes is
their formulation or modification with substances increasing
their capacity to stimulate T cells. These include T helper cell
epitopes, lipids or liposomes (or the preferred modifications as
described in WO 01/78767).

Another way to increase the T cell stimulating capacity of epi-
topes is their formulation with immune stimulating substances
for instance antibodies, cytokines or chemokines like interleuk-
in-2, -7, -12, -18, type I and II interferons (IFN), especially
IFN-alpha, GM-CSF, TNF-alpha, flt3-ligand and others.

According to a further aspect, the present invention is drawn to
the use of a HCV epitope or HCV peptide according to the present
invention for the preparation of a HLA restricted vaccine for

treating or preventing hepatitis C virus (HCV) infections.

A preferred HCV vaccine according to the present invention com-
prises at least two of the following epitopes:
KFPGGGQIVGGVYLLPRRGPRLGVRATRK, DLMGYIPAV, LEDRDRSELSPLLLSTTEW,
DYPYRLWHYPCTVNFTIFKV, GYKVLVLNPSVAAT, CINGVCWTV, AAWYELT-
PAETTVRLR, YLVAYQATVCARAQAPPPSWD, TAYSQQTRGLLG, HMWNFIS-
GIQYLAGLSTLPGNPA, IGLGKVLVDILAGYGAGVAGALVAFK and SMSYTWTGALITP.
Preferably, this HCV vaccine comprises at least four, preferably
at least five, at least six, at least eight, or all twelve of
these epitopes.

Another preferred HCV vaccine according to the present invention
comprises at least two of the following epitopes:
KFPGGGQIVGGVYLLPRRGPRLGVRATRK, DYPYRLWHYPCTVNFTIFKV
AAWYELTPAETTVRLR, TAYSQQTRGLLG, HMWNFISGIQYLAGLSTLPGNPA, IGLGK-
VLVDILAGYGAGVAGALVAFK and SMSYTWTGALITP. Preferably, this HCV
vaccine comprises at least four, at least five, especially all
seven of these epitopes.

The present HCV vaccine preferably comprises at least one A2
epitope and at least one DR1 epitope.

The present HCV vaccine preferably comprises at least one DR7
epitope.

The following combination of epitopes is regarded as specific-
ally powerful (at least one from at least three of the groups
(1) to (5)):

(1) KFPGGGQIVGGVYLLPRRGPRLGVRATRK or KFPGGGQIVGGVYLLPRRGPRL or
YLLPRRGPRLGVRATRK or YLLPRRGPRL or LPRRGPRL or, LPRRGPRLGVRATRK
or GPRLGVRATRK or RLGVRATRK or KFPGGYLLPRRGPRLGVRATRK,
(2) AYAAQGYKVLVLNPSVAAT or AYAAQGYKVL or AAQGYKVLVLNPSVAAT or
KVLVLNPSVAAT or GYKVLVLNPSVAAT or AYAAQGYKVLVLNPSV or AYAAQGYK-
VLVLNPSVAA or AAQGYKVLVLNPSVA or AYAAQGYKVLPSVAAT or AYAAQGYKV-
LAAT,
(3) DLMGYIP(A/L)VGAPL or DLMGYIPALVGAPL or DLMGYIP(A/L)VG or
DLMGYIP(A/L)VGAP or DLMGYIP(A/L)V or DLMGYIPLVGAPL or
DLMGYIPLVGA or DLMGYIPLV,

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(4) GEVQVVSTATQSFLATCINGVCWTV or GEVQVVSTATQSFLAT or CINGVCWTV
or VSTATQSFLATCINGVCWTV or TQSFLATCINGVCWTV or GEVQVVSTATQSFLAT-
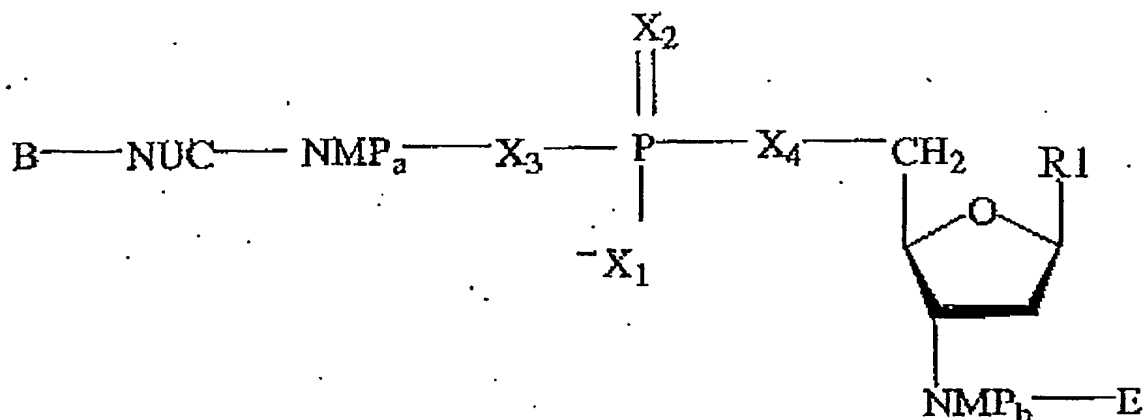CING or GEVQVVSTATQSFLAT,
(5) HMWNFISGIQYLAGLSTLPGNPA or MWNFISGIQYLAGLSTLPGNPA or HMWN-
FISGI or MWNFISGIQYLAGLSTLPGN or NFISGIQYLAGLSTLPGN or QYLAGLSTL
or HMWNFISGIQYLAGLSTL or HMWNFISGISTLPGNPA or HMWQYLAGLSTLPGNPA
or MWNFISGIQYLAGLSTLPGN; especially a HCV vaccine comprising the
epitopes GYKVLVLNPSVAAT, DLMGYIPAV, CINGVCWTV and HMWNFIS-
GIQYLAGLSTLPGNPA has been proven to be specifically powerful.

It is preferred to provide specifically suitable auxiliary sub-
stances for the present HCV vaccines. Therefore certain types of
immunostimulating substances have proven to be advantageous for
the present invention.

Therefore, a preferred HCV vaccine further contains a peptide
comprising a sequence $R_1-XZXZ_NXZX-R_2$, whereby N is a whole number
between 3 and 7, preferably 5, X is a positively charged natural
and/or non-natural amino acid residue, Z is an amino acid
residue selected from the group consisting of L, V, I, F and/or
W, and R_1 and R_2 are selected independantly one from the other
from the group consisting of -H, $-NH_2$, $-COCH_3$, -COH, a peptide
with up to 20 amino acid residues or a peptide reactive group or
a peptide linker with or without a peptide; $X-R_2$ may be an amide,
ester or thioester of the C-terminal amino acid residue of the
peptide ("Peptide A").

Another preferred embodiment of the HCV vaccine further contains
an immunostimulatory oligodeoxynucleic acid molecule (ODN) hav-
ing the structure according to the formula (I)

$$\text{B——NUC——NMP}_a\text{——X}_3\text{——}\overset{\displaystyle X_2}{\underset{\displaystyle {}^-X_1}{\overset{\displaystyle \|}{P}}}\text{——X}_4\text{——CH}_2\text{——R1}$$

NMP$_b$——E

wherein
R1 is selected from hypoxanthine and uracile,
any X is O or S,
any NMP is a 2' deoxynucleoside monophosphate or monothiophosphate, selected from the group consisting of deoxyadenosine-, deoxyguanosine-, deoxyinosine-, deoxycytosine-, deoxyuridine-, deoxythymidine-, 2-methyl-deoxyinosine-, 5-methyl-deoxycytosine-, deoxypseudouridine-, deoxyribosepurine-, 2-amino-deoxyribosepurine-, 6-S-deoxyguanine-, 2-dimethyl-deoxy-guanosine- or N-isopentenyl-deoxyadenosine-monophosphate or -monothiophosphate,
NUC is a 2' deoxynucleoside, selected from the group consisting of deoxyadenosine-, deoxyguanosine-, deoxyinosine-, deoxycytosine-, deoxyinosine-, deoxythymidine-, 2-methyl-deoxy-uridine-, 5-methyl-deoxycytosine-, deoxypseudouridine-, deoxyribosepurine-, 2-amino-deoxyribosepurine-, 6-S-deoxyguanine-, 2-dimethyl-deoxyguanosine- or N-isopentenyl-deoxyadenosine,
a and b are integers from 0 to 100 with the proviso that a + b is between 4 and 150, and
B and E are common groups for 5' or 3' ends of nucleic acid molecules ("I-/U-ODN").

A HCV vaccine composition according to the present invention may further comprise a polycationic compound, preferably a polycationic polymer, more preferably a polycationic peptide, especially polyarginine, polylysine or an antimicrobial peptide.

The polycationic compound(s) to be used according to the present

invention may be any polycationic compound which shows the char-
acteristic effect according to the WO 97/30721. Preferred polyc-
ationic compounds are selected from basic polypeptides, organic
polycations, basic polyaminoacids or mixtures thereof. These
polyaminoacids should have a chain length of at least 4 amino
acid residues. Especially preferred are substances containing
peptidic bonds, like polylysine, polyarginine and polypeptides
containing more than 20%, especially more than 50% of basic
amino acids in a range of more than 8, especially more than 20,
amino acid residues or mixtures thereof. Other preferred polyca-
tions and their pharmaceutical compositions are described in WO
97/30721 (e.g. polyethyleneimine) and WO 99/38528. Preferably
these polypeptides contain between 20 and 500 amino acid
residues, especially between 30 and 200 residues. These polyca-
tionic compounds may be produced chemically or recombinantly or
may be derived from natural sources.

Cationic (poly)peptides may also be polycationic anti-bacterial
microbial peptides. These (poly)peptides may be of prokaryotic
or animal or plant origin or may be produced chemically or re-
combinantly. Peptides may also belong to the class of defensins.
Such host defense peptides or defensins are also a preferred
form of the polycationic polymer according to the present inven-
tion. Generally, a compound allowing as an end product activa-
tion (or down-regulation) of the adaptive immune system, prefer-
ably mediated by APCs (including dendritic cells) is used as
polycationic polymer.

Polycationic substances used in the method according to the
present invention are cathelicidin derived antimicrobial pep-
tides or derivatives thereof (WO02/13857 A incorporated herein
by reference), especially antimicrobial peptides derived from
mammalian cathelicidins, preferably from human, bovine or mouse,
or neuroactive compounds, such as (human) growth hormone (as de-
scribed e.g. in WO01/24822).

Polycationic compounds derived from natural sources include HIV-
REV or HIV-TAT (derived cationic peptides, antennapedia pep-
tides, chitosan or other derivatives of chitin) or other pep-
tides derived from these peptides or proteins by biochemical or

recombinant production. Other preferred polycationic compounds
are cathelin or related or derived substances from cathelin, es-
pecially mouse, bovine or especially human cathelins and/or
cathelicidins. Related or derived cathelin substances contain
the whole or parts of the cathelin sequence with at least 15-20
amino acid residues. Derivations may include the substitution or
modification of the natural amino acids by amino acids which are
not among the 20 standard amino acids. Moreover, further cation-
ic residues may be introduced into such cathelin molecules.
These cathelin molecules are preferred to be combined with the
HCV peptide composition according to the present invention.
However, these cathelin molecules surprisingly have turned out
to be also effective as an adjuvant for a antigen without the
addition of further adjuvants. It is therefore possible to use
such cathelin molecules as efficient adjuvants in vaccine formu-
lations with or without further immunactivating substances.

Preferably, the present HCV vaccine further contains an $Al(OH)_3$
adjuvant and/or a polycationic peptide.

According to a specifically preferred embodiment, the above men-
tioned Peptide A is $KLKL_5KLK$ and/or the above mentioned I-/U-ODN
is oligo $d(IC)_{13}$.

In certain embodiments, it can be preferred that the HCV vaccine
further contains an oligodeoxynucleotide containing a CpG-motif.

Preferably, the HCV vaccine according to the present invention
is lyophilised in a form which is reconstitutable within 15 min.
at 37°C. Depending on the individual epitopes in the HCV vac-
cine, this may be a difficult task. Therefore, the present in-
vention also provides for efficient means for formulating such a
HCV vaccine.

The HCV vaccine according to the present invention preferably
contains between 10 µg and 10 mg of each epitope (per dose).

Depending on the application the number of the individual pep-
tides and especially their concentration in the mixture varies.
In a preferred embodiment of the present invention the concen-

tration of the individual peptides in the HCV vaccine to be de-
livered to the patient ranges from 5μg/ml to 5mg/ml, preferably
from 50μg/ml to 4mg/ml, more preferably from 100μg/ml to 3mg/ml.

The lyophilised HCV vaccine according to the present invention
preferably contains traces of acetic acid due to the lyophilisa-
tion process according to the present invention.

According to another aspect, the present invention also relates
to the use of the present HCV vaccine for the preparation of a
medicament for the prevention and treatment of an infection with
HCV, i.e. administering an effective immunising dose of a HCV
vaccine according to the present invention to an individual, es-
pecially a HCV patient or an individual being at risk of aquir-
ing a HCV infection (e.g. hospital personnel, clinical research
personnel, blood donation personnel, etc.).

Solubilising peptide mixtures such as the HCV vaccines according
to the present invention can be difficult. It is therefore an
aim of the present invention to provide methods for the solubil-
isation of the HCV peptide mixtures according to the present in-
vention, i.e. peptide mixtures which are composed of two or more
peptides, especially those consisting of hydrophilic as well hy-
drophobic peptides. Another aim is to make available a method
for the manufacturing of sterile and optionally lyophilised
pharmaceutical formulations containing peptide mixtures.

Therefore, the present invention provides according to another
aspect a method for making a pharmaceutical preparation compris-
ing the solubilisation of a HCV peptide mixture according to the
present invention, characterised in that the peptide mixture is
solubilised by an aqueous solution containing at least one or-
ganic acid selected from the group consisting of formic acid,
acetic acid, propionic acid, butyric acid and halogenated or hy-
droxylated forms thereof. In order to elongate the shelf life
the solubilised peptide mixture may be further sterilised (by
filtration, irradiation, heat treatment, chemical sterilisation
or other methods) and lyophilised.

In a preferred embodiment the peptide mixture contains hydro-

philic as well hydrophobic peptides. Of course the present meth-
od is also usable for peptide mixtures containing only one type
of peptides (specifically for mixtures containing e.g. two or
more hydrophilic peptides).

In another preferred embodiment the peptides which are dissolved
by an aqueous solution according to the present invention con-
tain at least 6, preferably at least 8, more preferably at least
10, especially at least 12 amino acids. Peptides as well poly-
peptides containing a maximum number of 30, 40 or 50 (or even up
to 100, although long epitopes are less preferred due to the
disadvantages of longer antigen fragments) amino acids can be
dissolved according to the present invention.

For this aspect of the present invention peptides are character-
ised by their solubility. Peptides which are soluble in an
aqueous solution less than 100µg/ml are considered hydrophobic.
On the other hand hydrophilic peptides dissolve in a buffered
aqueous solution in concentration over 100µg/ml. Hydrophilic
peptides may further be divided into cationic (>20% basic amino
acids including lysine, arginine, histidine), anionic (>20%
acidic amino acids including aspartic acid, glutamic acid) and
hydroxyl-rich peptides (>30% -OH containing amino acids like
serine, threonine, tyrosine).

According to another aspect, the present invention therefore
relates to a method for the preparation of the present HCV vac-
cine which is characterised by the following steps:
-     chemically synthesising the at least two epitopes as
defined above,
-     solubilising these epitopes by an aqueous solution contain-
      ing at least one organic acid selected from the group con-
      sisting of formic acid, acetic acid, propionic acid, butyric
      acid and halogenated or hydroxylated forms thereof,
-     mixing the solubilised epitopes and
-     optionally lyophilising the mixed epitopes.

The HCV peptide mixture can be deep frozen (if no lyophilisation
step is carried out) for storage reasons.

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The peptides of the peptide mixture according to the present in-
vention are synthesised by standard chemical methods (e.g. solid
phase synthesis according to Merrifield). Of course the peptides
may also be obtained by chemical or enzymatic fragmentation of
recombinant produced or native isolated proteins. In another em-
bodiment the HCV peptides are directly isolated from eukaryotic
and prokaryotic cells. In all three cases additional purifica-
tion of the peptide or polypeptide of interest will in some
cases be required before they are lyophilised and mixed togeth-
er.

It is a basic requirement for this specific method that the or-
ganic acids used in aqueous solutions for the solubilisation of
peptides are pharmaceutically acceptable. In a preferred embodi-
ment of the present invention the organic acids are acetic acid
and/or formic acid containing optionally acetonitrile.

The experiments revealed, depending on the composition of the
peptide mixture, that the concentration of the organic acid in
the aqueous solution has to be at least 10%, preferably at least
20%, preferably at least 30%, preferably at least 40%, prefer-
ably at least 50%, preferably at least 60%.

In some cases the addition of derivatives of organic acids (e.g.
acetonitrile) to the aqueous solution may be helpful to solubil-
ise peptide mixtures containing a larger quantity of hydrophobic
peptides. Also organic solvents such as DMSO and DMF contribute
to an enhanced dissolution of peptide mixtures containing an in-
creased concentration of hydrophobic peptides. However, aqueous
peptide mixtures containing DMSO and/or DMF cannot be freeze-
dried.

In a preferred embodiment bulking agents are added to the pep-
tide mixture when the product is intended to be lyophilised. Es-
pecially at low peptide concentrations the forming of a good ly-
ophilisation cake is not guaranteed. Therefore at low amounts of
peptides bulking agents are added. Preferred bulking agents are
sorbitol, mannitol, polyvinylpyrrolidone (PVP) and mixtures
thereof.

In another preferred embodiment the solubilised peptide mixture
is sterilised by filtration. In order to get an increased recov-
ery of the product and to allow filtration of large amounts of
the peptide mixture, the containing peptides have to be com-
pletely solubilised and no gel may be formed.

The solubilised and optionally sterilised peptide mixture can be
lyophilised directly or after filling into vials. Lyophilisation
is a useful and effective method to prolong the shelf life of
peptide mixtures as described above. With the solubilised pep-
tides a lyophilised preparation of a mixture of peptides, con-
taining a maximum of 5% of residual solvent (water in aqueous
systems) and traces of the organic acid, can be obtained which
is reconstitutable in a buffered aqueous solution containing
NaCl and/or sorbitol within 10 minutes of >95%, preferably of
98%, especially of 99%, resulting in a turbid suspension or
clear solution (depending on the composition of the peptide mix-
ture). Such a quick reconstitution is specifically necessary in
emergency cases, where a ready-to-use solution has to be present
within a few minutes with an almost complete re-solvation of the
whole dosis of a lyophilised vial.

The invention will be described in more detail by the following
examples and figures, but the invention is of course not limited
thereto.

Fig. 1 shows that KLK/O-d(IC)$_{13}$ induces HCV-peptide specific type
1 cellular immune responses;

EXAMPLES:

**Example I. Binding of HCV derived peptides to HLA class II mo-
lecules**
According to the WO04/024182, several new peptides incorporating
sequences from overlapping reactive HCV peptides or avoiding
critical residues like cystein were synthesised. These were re-
tested for their affinities to class II soluble HLA molecules,
and results were compared to those obtained with the original
(Table 1).

Table 1. Binding of selected HCV-derived peptides and their 15-mer counterparts to soluble HLA class II molecules ("+++" strong affinity, "++" intermediate affinity, "+" weak affinity, "-" no affinity, "nd" not done; core binding motifs are underlined).

Peptide ID	Peptide sequences	Binding to soluble HLA-DRB1*				
		0101	0401	0404	0701	1101
1798	IGLGKVLVDILAGYGAGVAGALVAFK	-	-	+	++	+/-
B84	GSIGLGKVLVDILAG	+	+	+	-	
B86	IGLGKVLVDILAGYG	+	++	+	+	+/-
B88	LGKVLVDILAGYGAG	+	++	+		
B92	LVDILAGYGAGVAGA	+	-			
B94	DILAGYGAGVAGALV	+	-	-	-	
B96	LAGYGAGVAGALVAF	++	++	-	+/-	+/-
1799	AAWYELTPAETTVRLR	+++	+	+	-	+/-
B46	AGAAWYELTPAETTV	+++	+++	+++	-	+/-
B48	AAWYELTPAETTVRL	+++	+++	+++	-	+/-
1827	TAYSQQTRGLLG	++	-	+/-	+	+
C114	TAYSQQTRGLLGCIV	+++	+/-	+/-	+	++
1829	SMSYTWTGALITP	+	-	-	+	+/-
1604	VVCCSMSYTWTGALITPC	+	+	++	++	+
1650	VDYPYRLWHYPCTVNFTIFKVRMYVGGVEHRL					
A130	DYPYRLWHYPCTVNF	+	++	+/-		
A131	YPYRLWHYPCTVNFT	-				
A135	LWHYPCTVNFTIFKV	-	-		++	
A141	TVNFTIFKVRMYVGG	-	-	+/-		++
A145	TIFKVRMYVGGVEHR	+/-	-			
1651	VDYPYRLWHYPCTVNYTIFKIRMYVGGVEHRL					
1800	DYPYRLWHYPCTVNYTIFKI	-	-	+/-	++	-
A147	DYPYRLWHYPCTVNY	-	-			
A152	LWHYPCTVNYTIFKI	-	-			
A158	TVNYTIFKIRMYVGG	-	-	+/-		
A162	TIFKIRMYVGGVEHR	+/-	-			

1817		RMYVGGVEHRL	-	-	+/-		
1426	HMWNFISGIQYLAGLSTLPGNPA		+	+	++	++	+
1425	NFISGIQYLAGLSTLPGNPA		++	++	++	nd	nd
1006	MWNFISGIQYLAGLSTLPGN		++	+	++	nd	nd

Abolished affinities to DRB1*0101 and DRB1*0401 molecules in the case of peptide 1798 in comparison with its shorter counterparts (B84 - B96) is probably due to the long sequence (26 amino acids) which can have a secondary structure that prevents binding. It is to be expected that in vivo, upon proteolytic cleavage, peptide 1798 will give rise to two shorter class II epitopes. Removed cystein (C) residues in peptides 1827 and 1829 (derivatives of peptides C114 and 1604, respectively) seem to be crucial for binding to DRB1*0401 molecules but do not essentially change affinities to other tested DR subtypes.

Example II. Identification and Characterisation of HCV-epitope hotspots

As outlined above, a T-cell epitope hotspot (a "hotspot") is defined as a short peptide sequence at least comprising more than one T-cell epitope. For example, two or more epitopes may be located shortly after each other (shortly being defined as less than 5-10 amino acids), or directly after each other, or partially or even fully over-lapping. Hotspots may contain only class I or class II epitopes, or a combination of both. Epitopes in hotspots may have different HLA restrictions.

Due to the highly complex and selective pathways of class I and class II antigen processing, referred to in the introduction, T-cell epitopes cannot be easily predicted within the sequence of a polypeptide. Though widely used, computer algorithms for T-cell epitope prediction have a high rate of both false-negatives and false-positives.

Thus, as even individual T-cell epitopes are not obvious within the sequence of a polypeptide, the same is even more the case for hotspots. Several radically different experimental ap-

proaches are combined according to the present invention for T-
cell epitope identification, including epitope capture, HLA-
transgenic animals and *in vitro* stimulation of human mononuclear
cells. All three approaches are systematically applied on over-
lapping peptides spanning the antigen of interest, enabling com-
prehensive identification of epitopes. Upon such a comprehensive
analysis, not limited to a particular HLA allele, but rather un-
ravelling all possibly targeted epitopes within a population,
epitope hotspots may become apparent. Within an antigen, only
few if any sequences show characteristics of hotspots. Thus the
identification of a hotspot is always a surprising event.

T-cell epitope hotspots offer important advantages: Hotspots can
activate and can be recognised by different T-cell clones of a
subject. Hotspots (when comprising epitopes with different HLA
restriction) can interact with T-cells from different non HLA-
matched individuals.

Epitope-based vaccines, so far have aimed at selected prevalent
HLA-alleles, for instance HLA-A2, which is expressed in about
half of Caucasians. Since other alleles are less frequent, epi-
tope-based vaccines with broad worldwide population coverage
will have to comprise many different epitopes. The number of
chemical entities (for instance peptides) of a vaccine is lim-
ited by constraints originating from manufacturing, formulation
and product stability.

Hotspots enable such epitope-based vaccines with broad worldwide
population coverage, as they provide a potentially high number
of epitopes by a limited number of peptides.

Table 2: T-cell epitope hotspots in conserved regions of HCV.
Hotspots (incl. some variations) are shown in bold, epitopes
contained within the hotspots in normal font. Peptide number and
sequence, as well as HLA-class I and class II coverage are giv-
en. Source data refers to literature references, if not provided
by WO04/024182.

peptide ID	peptide sequence	class I	class II	source data (besides WO04/024182)
1835	KFPGGGQIVGGVYLLPRRGPRLGVRATRK	A2, A3, B7	DR11	

83	KFPGGGQIVGGVYLLPRRGPRL	A2	B7	DR11	
1051	YLLPRRGPRL	A2			Bategay 1995
1843	LPRRGPRL	B7			Example III
	GPRLGVRAT	B7			Koziel 1993
	RLGVRATRK	A3			Chang 1999
84	GYKVLVLNPSVAAT			DR1,4,7,11	
	AYAAQGYKVL	A24			prediction
84EX	AYAAQGYKVLVLNPSVAAT	A24		DR1,4,7,11	
87	DLMGYIPAV	A2			Sarobe 1998
	GYIPLVGAPL	A24			prediction
87EX	DLMGYIPLVGAPL	A2,A24			
89	CINGVCWTV	A2			Koziel 1995
1577	GEVQVVSTATQSFLAT			DR 4, 7	
89EX	GEVQVVSTATQSFLATCINGVCWTV	A2		DR 4, 7	
1426	HMWNFISGIQYLAGLSTLPGNPA	A2		DR1,4,7,11	
1006	MWNFISGIQYLAGLSTLPGN				
1425	NFISGIQYLAGLSTLPGNPA				
	QYLAGLSTL	A24			prediction
1334	HMWNFISGI	A2			Wentworth 1996
1650	VDYPYRLWHYPCTVNFTIFKVRMYVGGVEHRL	Cw7,A2,A24, A11,A3		DR1,4,7,11	
1836	DYPYRLWHYPCTVNFTIFKI	Cw7,A2;A24, A11		DR1,4,7,11	
1846	DYPYRLWHYPCTVNFTIFKV	Cw7,A2;A24, A11		DR1,4,7,11	
1651	VDYPYRLWHYPCTVNYTIFKIRMYVGGVEHRL				
1800	DYPYRLWHYPCTVNYTIFKI	Cw7,A24,A11		DR7	
1754	DYPYRLWHY	Cw7			Lauer 2002
1815	TVNYTIFKI	A11			prediction
1816	TINYTIFK	A11			Koziel 1995
	TVNFTIFKV	A11			prediction
	HYPCTVNYTI	A24			prediction
	HYPCTVNFTI	A24			prediction
	RMYVGGVEHR	A3			Chang 1999
1799	AAWYELTPAETTVRLR	B7? B35		DR1, 4	
1818	TPAETTVRL	B7? B35			Ibe 1998
1827EX	GWRLLAPITAYSQQTRGLLGCIV	A2,A3,A24, B8		DR1,4,7,11	

C114	TAYSQQTRGLLGCIV	A24, B8?	DR1,4,7,11	
1827	TAYSQQTRGLLG	A24, B8	DR1, 7,11	
C112	GQGWRLLAPITAYSQ	A3?,A2?,	DR1	
	RLLAPITAY	A3		prediction
C114EX	GQGWRLLAPITAYSQQTRGLLGCIV	A24,A3?,A2?, B8?	DR1,4,7,11	
1827EX	GQGWRLLAPITAYSQQTRGLLG	A24,A3?,A2?, B8?	DR1, 7,11	
1801	AYSQQTRGLL	A24		
1819	AYSQQTRGL	A24		Kurokohchi 2001
1798	IGLGKVLVDILAGYGAGVAGALVAFK	A2,24,3,11	DR1,4,7	
1820	ILAGYGAGV	A2		Bategay 1995
1821	VAGALVAFK	A3,11		Chang 1999
	GYGAGVAGAL	A24		prediction
1604	VVCCSMSYTWTGALITPC	A2,A24,B7	DR1,4,7,11	
1829	SMSYTWTGALITP	A2,A24,B7,	DR1, 7,11	
	SMSYTWTGAL	A2,B7		prediction
	SYTWTGALI	A24		prediction
1579	FTDNSSPPAVPQTFQV	A1,2;B7,51	DR53=B4*01	Tab.5
1624	LEDRDRSELSPLLLSTTEW	A1,2,3,26 B8,27,4402,60	DR7	
1848	LEDRDRSELSPLLLST	A1,2,3,26, B8,27,4402,60	DR7	
	RSELSPLLL	A1		prediction
	ELSPLLLST	A2,A3		prediction
	DRDRSELSPL	A26,B27		prediction
	LEDRDRSEL	B08,B4402		prediction
1824	LEDRDRSEL	B60		Wong 2001
1547	YLVAYQATVCARAQAPPPSWD	A2	DR1,4,7,11	
1822	YLVAYQATV	A2		Wentworth 1996
A1A7	MSTNPKPQRKTKRNTNR	A11,B08,B27		
A3A7	PQRKTKRNTNR	B08,B27		
	QRKTKRNTN	B08		prediction
	RKTKRNTNR	B2705		prediction
	MSTNPKPQR	A11		prediction
	MSTNPKPQK	A11		Wong 1998
A122EX	LINTNGSWHINRTALNCNDSL	A2,2,3,B8	DR1,4,7,11	
A122	NGSWHINRTALNCNDSL	A2	DR1,4,7,11	
	LINTNGSWHI	A2,3		prediction

	RTALNCNDSL	A2		prediction
1825	LINTNGSWHINRTALN	A2,3,B8		prediction
1826	SWHINRTALN	B8		prediction
A241	TTILGIGTVLDQAET	A2,A3	DR1, 4	
	TTILGIGTV	A2		prediction
	TILGIGTVL	A3		prediction
B8B38	FDSSVLCECYDAGAAWYE	A1,2,3,26		
B8	FDSSVLCECYDAGCA	A3,A26		
	VLCECYDAGA	A2		prediction
B38	VVLCECYDAGAAWYE	A1		
C70EX	ARLIVFPDLGVRVCEKMALY	A2,A3,B27		
C64-C70	ARLIVFPDL	B*2705?,*2709?		
1831	RLIVFPDLGV	A2		Gruener 2000
1832	RVCEKMALY	A3		Wong 1998
C92	AFCSAMYVGDLCGSV	A2,B51	DR1,4	
C97	GVLFGLAYFSMVGNW	A2,3,26, B2705,51	DR1,4,7	
C106	TRVPYFVRAQGLIRA	A3,24, B7,B8,B2705	DR1,4,7	
C134	TTLLFNILGGWVAAQ	A2	DR1, 7,11	
1823	LLFNILGGWV	A2		Bategay 1995

Example III. HCV epitope hotspot Ipep 1426 contains at least HLA-A*0201 and several promiscuous class II T-cell epitopes

The major objective of this experiment was to compare the immunogenicity of the "hospot" Ipep 1426, which contains at least one HLA-A*0201 epitope (Ipep 1334) and 2 promiscuous class II epitopes (Ipeps 1006 and 1425), to the individual epitopes. To this end peripheral blood mononuclear cells (PBMC) from several healthy HLA-typed blood donors were stimulated in vitro either with 1426 or a mixture of 1334, 1006, 1425. Three rounds of stimulation were performed resulting in oligoclonal T cell lines. Then, responses against all four peptides were assessed by interferon-gamma (IFN-γ) ELIspot analysis.

Peptide 1426, induces T cell responses similarly well as individual epitopes comprised within its sequence. In particular, CD8 positive T cells directed against the HLA-A*0201 restricted epi-

tope 1334 were successfully generated.

Table 3: peptide induced IFN-γ secretion of oligoclonal T cell lines. Lines were generated from two HLA-typed healthy individuals by 3 rounds of in vitro priming with either peptide 1426 or a mixture of peptides 1006+1425+1334. The reactivity of CD4 and CD8 positive T cells in these lines was assessed by IFN-γ ELIspot ("+++" very strong, "++" strong, "+" significant, "-" no IFN-gamma secretion).

Donor HLA	A*0201, A*03, B7, B60; DRB1*1501, −B1*1302		A*0206, A*01, B27, B50; DRB1*0401, −B1*1402	
Peptide ID	line raised against 1426	line raised against 1006+1425+1334	line raised against 1426	line raised against 1006+1425+1334
1006	++	++	++	++
1425	+++	+++	+++	++
1334	+	+	−	−
1006+1425+1334	++	++	++	++
1426	+++	+++	+++	++
84 (HCV derived negative control)	−	−	−	−

Example IV. Potent HCV-specific type 1 cellular responses are induced by the combined injection of five different HCV-derived peptides, the antimicrobial peptide KLK and the synthetic oligodeoxynucleotide o-d(IC)$_{13}$.

Mice HLA-A*0201 transgenic mice (HHD.1)

Peptides   The peptides p83, p84, p87, p89, p1426

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were used for vaccination.

p83: HCV-derived peptide,
    (KFPGGGQIVGGVYLLPRRGPRL)

p84: HCV-derived peptide,
    (GYKVLVLNPSVAAT)

p87: HCV-derived peptide,
    (DLMGYIPAV)

p89: HCV-derived peptide,
    (CINGVCWTV)

p1426: HCV-derived peptide,
    (HMWNFISGIQYLAGLSTLPGNPA)

(p1274 used for restimulation as irrelev-
ant peptide
(YMDGTMSQV; HLA-A*0201 restricted)

All peptides were synthesised by standard
solid phase F-moc synthesis, HPLC purified
and analyzed by mass spectroscopy for pur-
ity.

dose: 20µg per peptide/mouse

KLK   KLKLLLLLKLK-COOH was synthesised by MPS (Multiple Peptide
System, USA)

dose: 10nmol/mouse

oligo-d(IC)$_{13}$ (= ODN1a) ODN 5'ICI CIC ICI CIC ICI CIC ICI CIC IC3'
was synthesised by Purimex Nucleic Acids
Technology, Göttingen

dose: 0.4nmol/mouse

Formulation   10mM Tris/135mM NaCl; pH ~7

Experimental setup (5 mice/group):

    1. HCV peptides
    2. HCV peptides + KLK + o-d(IC)$_{13}$

On days 0, 14 and 28 HHD.1 mice were injected s.c. into both hind footpads with a total volume of 100μl/mouse (50μl/footpad) containing the above listed compounds. At day 35 (7 days after last vaccination) CD4$^+$ as well as CD8$^+$ T cells were isolated by magnetic separation (MACS, Miltenyi) from single cell suspensions of spleen cells. T cells were incubated with medium (background control) or were restimulated with irradiated spleen cells from naïve HHD.1 mice as APC in the presence of either the different peptides used for vaccination or the irrelevant peptide p1274. After overnight incubation, the IFN-γ production was analyzed using an ELIspot assay.

Figure 1 shows that upon co-injection of the five HCV-derived peptides with KLK/o-d(IC)$_{13}$ high amounts of IFN-γ produced by CD4$^+$ T cells against p84, p87, p89, p1426 were induced. Furthermore, a strong IFN-γ production by CD8$^+$ T cells against p87, p89 was detectable.

Example V: Solubilisation of peptide mixtures (HCV peptides plus poly-L-arginine):

The peptide mixture contains the peptides 83, 84, 87, 89, 1426 and pR as immuniser (see table 4), wherein peptides 83 and 84 are soluble in water and DMSO, peptides 87 and 89 are poorly water soluble, but dissolve easily in DMSO and peptide 1426 is

only soluble in DMSO. For the manufacturing of a suspension for-
mulation it is necessary first to dissolve peptides 83 and 84 in
an aqueous buffer solution and peptides 87, 89, and 1426 in
DMSO. After sterile filtration the solutions can be combined to
form the final suspension.

Water/acetonitrile mixtures as well as DMSO and DMF did not work
as solvents and resulted in suspensions that could not be
sterile filtered due to filter blockage. Surprisingly a 50%
acetic acid/water mixture was found to dissolve all components.
The obtained peptide solution containing the above mentioned
peptides could easily sterile filtered without any filter block-
ing.

Table 4 Peptide mixture I

Peptide ID	Peptide sequence
83	KFPGGGQIVGGVYLLPRRGPRL
84	GYKVLVLNPSVAAT
87	DLMGYIPAV
89	CINGVCWTV
1426	HMWNFISGIQYLAGLSTLPGNPA

The following examples (examples V.1 to V.12, tables 5 to 16)
illustrate that peptide mixtures containing at least two pep-
tides with variable length and amino acid composition are suit-
able to be solubilised according to the present invention. Fur-
thermore the tables show the known affinities of the peptides to
HLA class I and class II molecules.

Example V.1:

Table 5 Peptide mixture II

Peptide ID	Peptide sequence	HLA class I	HLA class II
1835	KFPGGGQIVGGVYLLPRRGPRLGVRATRK	A2, A3, B7	DR11
87	DLMGYIPAV	A2	
1624	LEDRDRSELSPLLLSTTEW	B60	DR7

Peptide ID	Peptide sequence	HLA class I	HLA class II
1846	DYPYRLWHYPCTVNFTIFKV	A2, A11, Cw7	DR1, 4, 7, 11
84	GYKVLVLNPSVAAT		DR1, 4, 7, 11
89	CINGVCWTV	A2	
1799	AAWYELTPAETTVRLR	B35	DR1, 4
1547	YLVAYQATVCARAQAPPPSWD	A2	DR1, 4, 7, 11
1827	TAYSQQTRGLLG	A24	DR1, 7, 11
1426	HMWNFISGIQYLAGLSTLPGNPA	A2	DR1, 4, 7, 11
1798	IGLGKVLVDILAGYGAGVAGALVAFK	A2, A3, A11	DR1, 4, 7
1829	SMSYTWTGALITP	A2, B7	DR1, 7, 11

Example V.2:

Table 6 Peptide mixture III

Peptide ID	Peptide sequence	HLA class I	HLA class II
1835	KFPGGGQIVGGVYLLPRRGPRLGVRATRK	A2, A3, B7	DR11
1846	DYPYRLWHYPCTVNFTIFKV	A2, A11, Cw7	DR1, 4, 7, 11
1799	AAWYELTPAETTVRLR	B35	DR1, 4
1827	TAYSQQTRGLLG	A24	DR1, 7, 11
1426	HMWNFISGIQYLAGLSTLPGNPA	A2	DR1, 4, 7, 11
1798	IGLGKVLVDILAGYGAGVAGALVAFK	A2, A3, A11	DR1, 4, 7
1829	SMSYTWTGALITP	A2, B7	DR1, 7, 11

Example V.3:

Table 7 Peptide mixture IV

Peptide ID	Peptide sequence	HLA class I	HLA class II
C134	TTLLFNILGGWVAAQ	A2	DR1, 7, 11
1815	TVNYTIFKI	A11	

Peptide ID	Peptide sequence	HLA class I	HLA class II
1006	MWNFISGIQYLAGLSTLPGN		

Example V.4:

Table 8 Peptide mixture V

Peptide ID	Peptide sequence	HLA class I	HLA class II
84	GYKVLVLNPSVAAT		DR1, 4, 7, 11
87	DLMGYIPAV	A2	
89	CINGVCWTV	A2	
1426	HMWNFISGIQYLAGLSTLPGNPA	A2	DR1, 4, 7, 11

Example V.5:

Table 9 Peptide mixture VI

Peptide ID	Peptide sequence	HLA class I	HLA class II
1051	YLLPRRGPRL	A2	
84	GYKVLVLNPSVAAT		DR1, 4, 7, 11
87	DLMGYIPAV	A2	
89	CINGVCWTV	A2	
1426	HMWNFISGIQYLAGLSTLPGNPA	A2	DR1, 4, 7, 11

Example V.6:

Table 10 Peptide mixture VII

Peptide ID	Peptide sequence	HLA class I	HLA class II
1006	MWNFISGIQYLAGLSTLPGN		
1827EX	GWRLLAPITAYSQQTRGLLGCIV	B8	

Example V.7:

Table 11 Peptide mixture VIII

Peptide ID	Peptide sequence	HLA class I	HLA class II
1604	VVCCSMSYTWTGALITPC		
83	KFPGGGQIVGGVYLLPRRGPRL	B8	
A130	DYPYRLWHYPCTVNF		
B46	AGAAWYELTPAETTV		

Example V.8:

Table 12 Peptide mixture IX

Peptide ID	Peptide sequence	HLA class I	HLA class II
B84	GSIGLGKVLVDILAG		DR1, 4
B96	LAGYGAGVAGALVAF		DR1, 4, 7, 11
1829	SMSYTWTGALITP	A2, B7	DR1, 7, 11
A135	LWHYPCTVNFTIFKV		DR7
A130	DYPYRLWHYPCTVNF		DR1, 4
1426	HMWNFISGIQYLAGLSTLPGNPA	A2	DR1, 4, 7, 11
1817	RMYVGGVEHRL		DR4
87EX	DLMGYIPLVGAPL	A2, 24	
1816	TINYTIFK	A11	

Example V.9:

Table 13 Peptide mixture X

Peptide ID	Peptide sequence	HLA class I	HLA class II
1650	VDYPYRLWHYPCTVNFTIFKVRMYVG-GVEHRL	Cw7,A2,A24, A11, A3	DR1,4,7,11
1836	DYPYRLWHYPCTVNFTIFKI	Cw7,A2,A24, A11	DR1, 4, 7, 11

Peptide ID	Peptide sequence	HLA class I	HLA class II
1846	DYPYRLWHYPCTVNFTIFKV	Cw7, A2, A24, A1, A11	DR1,4,7,11
1651	VDYPYRLWHYPCTVNYTIFKIRMYVG-GVEHRL		
1800	DYPYRLWHYPCTVNYTIFKI	Cw7,A24,A11	DR7

Example V.10:

Table 14 Peptide mixture XI

Peptide ID	Peptide sequence	HLA class I	HLA class II
1835	KFPGGGQIVGGVYLLPRRGPRLGVRATRK	A2, A3, B7	DR11
83	KFPGGGQIVGGVYLLPRRGPRL	A2 B7	DR11
84EX	AYAAQGYKVLVLNPSVAAT	A24	DR1,4,7,11
87EX	DLMGYIPLVGAPL	A2,A24	
89EX	GEVQVVSTATQSFLATCINGVCWTV	A2	DR 4, 7
1426	HMWNFISGIQYLAGLSTLPGNPA	A2	DR1,4,7,11

Example V.11:

Table 15 Peptide mixture XII

Peptide ID	Peptide sequence	HLA class I	HLA class II
C114	TAYSQQTRGLLGCIV	A24, B8	DR1,4,7,11
1798	IGLGKVLVDILAGYGAGVAGALVAFK	A2,24,3,11	DR1,4,7
1604	VVCCSMSYTWTGALITPC	A2,A24,B7	DR1,4,7,11
1624	LEDRDRSELSPLLLSTTEW	A1,2,3,26	DR7

Example V.12:

Table 16 Peptide mixture XIII

Peptide ID	Peptide sequence	HLA class I	HLA class II
1547	YLVAYQATVCARAQAPPPSWD	A2	DR1,4,7,11
A1A7	MSTNPKPQRKTKRNTNR	A11,B08,B27	

Peptide ID	Peptide sequence	HLA class I	HLA class II
A122EX	LINTNGSWHINRTALNCNDSL	A2,2,3,B8	DR1,4,7,11
A241	TTILGIGTVLDQAET	A2,A3	DR1, 4
B8B38	FDSSVLCECYDAGAAWYE	A1,2,3,26	
C70EX	ARLIVFPDLGVRVCEKMALY	A2,A3,B27	
C92	AFCSAMYVGDLCGSV	A2,B51	DR1,4
C97	GVLFGLAYFSMVGNW	A2,3,26, B2705, 51	DR1,4,7
C106	TRVPYFVRAQGLIRA	A3,24,B7,B8,B2705	DR1,4,7
C134	TTLLFNILGGWVAAQ	A2	DR1, 7,11

Example VI. Rationale in designing further HCV vaccine variants according to the present invention

Table 17: preferred combination of "hotspots I"

Peptide ID	Peptide sequence	HCV antigen	HLA alleles	
			HLA class I	HLA class II
1835	KFPGGGQIVGGVYLLPRRGPRLGVRATRK	core	A2, A3, B7	DR11
87	DLMGYIPAV	core	A2	
1624	LEDRDRSELSPLLLSTTEW	E2	B60	DR7
1846	DYPYRLWHYPCTVNFTIFKV	E2	A2, A11, Cw7	DR1, 4, 7, 11
84	GYKVLVLNPSVAAT	NS3		DR1, 4, 7, 11
89	CINGVCWTV	NS3	A2	
1799	AAWYELTPAETTVRLR	NS3	B35	DR1, 4
1547	YLVAYQATVCARAQAPPPSWD	NS3	A2	DR1, 4, 7, 11
1827	TAYSQQTRGLLG	NS3	A24	DR1, 7, 11
1426	HMWNFISGIQYLAGLSTLPGNPA	NS4	A2	DR1, 4, 7, 11
1798	IGLGKVLVDILAGYGAGVAGALVAFK	NS4	A2, A3,A11	DR1, 4, 7
1829	SMSYTWTGALITP	NS5	A2, B7	DR1, 7, 11

Characteristics of preferred combination of hotspots I:
Number of peptides: 12
Potential interactions of peptides via Cysteins: 4 Cys in 3 pep-
tides (1846, 89, 1547); potential formation of homo- and het-
erodimers and potentially oligomers of peptides.
Number HCV antigens covered: 5 (core, E2, NS3, NS4, NS5)
HLA class I alleles covered: at least 8
A2 (8 times), A3 (2 times), A11 (2 times), A24 (1 time)
B7 (2 times), B35 (1 time), B60 (1 time)
Cw7 (1 time)
HLA class II covered: multiple by promiscuous peptides
     DR1 (8 times), DR4 (6 times), DR7 (8 times), DR11 (7 times)

The preferred combination of hotspots I consists of 12 peptides
derived from conserved regions (>80% in genotypes 1, 2, 3) of 5
different HCV antigens. In total at least 8 important HLA class
I alleles are covered (1-8 times, each), providing a population
coverage of 83-91% in Europe, 89-91% in U.S. Caucasians and 87-
89% in Japanese people (estimated on numbers from HLA-prevalence
studies in Gjertson and Terasaki, eds., HLA 1998, American Soci-
ety of Histocompatibility and Immunogenetics, Lenexa, Kansas,
USA). Similarly, several important HLA class II alleles are
covered, mostly by so called promiscuous peptides (binding to
more than one HLA class II allele). Individual particularly pre-
valent alleles (DR1, 4, 7, 11) are independently covered 6-8
times. Importantly, DR11 is covered 7 times. Presence of this
allele has been linked to spontaneous recovery from HCV infec-
tion (Thursz et al., 1999 Lancet: 354(9196):2119-24).
The high number of epitopes contained in this combination of
hotspots ensures not only broad population coverage, and hence
applicability of the vaccine, but also a broad immune response
within individuals treated with such a combination. This is a
pre-requisite for efficacy, since spontaneous recovery from HCV
infection has been linked to a broad immune response typically
directed against 10 or more epitopes (Day et al., 2002 J Virol.:
76(24):12584-95).

A T-cell response against multiple epitopes also reduces the

risk of development of HCV-epitope-escape variants (HCV viruses,
which do not contain the epitope targeted by the immune response
anymore), because the more epitopes targeted at the same time,
the more difficult for the virus to change them simultaneously.
Experiments in chimpanzees have established, that such HCV es-
cape variants are an important mechanism to maintain chronic in-
fection (Weiner et al, 1995 Proc Natl Acad Sci U S A.: 92(7):
2755-9).

Finally, targeting most of the HCV antigens (Core, E2, NS3, 4,
5) instead of for instance only one also provides significant
advantages: different antigens may be recognised differently by
T-cells, for instance due to differences in antigen processing,
or dictated by the particular HLA haplotype of the person).
Also, different antigens have different functional importance
during the HCV life-cycle.

Combination hotspot I contains also individual epitopes derived
from hotspots (Ipep87 derived from Ipep87EX and Ipep89 derived
from Ipep89EX). This has the advantage of a deliberate focusing
of the desired response on a particular epitope. Using for in-
stance Ipep89 guarantees that an HLA-A2 restricted CTL response
is generated, whereas using 89EX may leed to an HLA-A2 restric-
ted CTL response, that may be compromised by a concomitant DR4
or DR7 restricted T-helper response.

Using combinations of hotspots has a number of advantages over
using full-length antigens, either in the form of recombinant
proteins or as DNA vaccines. As detailed in the preceding para-
graph in certain cases using individual epitopes may be advant-
ageous over using the hotspot or the whole antigen. Second, the
hotspots disclosed herein have been identified via functional
assays using primarily a Th1/Tc1-type reaction (i.e. interferon
gamma) as read-out. It is well known that antigens do not only
contain such agonist epitopes, but may also encode antagonist
ligands. Such antagonists potentially present in whole antigens,
but absent in hotspots may impede immunogenicity and efficacy of
the vaccine. Next, whole antigens will almost certainly induce a
humoral immune response. Such an antibody response has per se
little effect on HCV residing within cells, but it can severely

compromise the desired T-cell reponse. Hotspots, due to their
restricted length, have a much lower probability to induce un-
wanted antibody responses. Finally, only relatively small parts
of HCV antigens are conserved between different genotypes. Thus,
whole antigens used in a vaccine may induce responses against
non-conserved regions or epitopes, irrelevant for most of the
patients. In contrast, the hotspots disclosed herein are derived
from regions conserved > 80% among the major HCV genotypes 1, 2
and 3.

Table 18: preferred combination of "hotspots II"

Pep-tide ID	Peptide sequence	HCV anti-gen	HLA alleles	
			HLA class I	HLA class II
1835	KFPGGGQIVGGVYLLPRRGPRLGVRATRK	core	A2, A3, B7	DR11
1846	DYPYRLWHYPCTVNFTIFKV	E2	A2, A11, Cw7	DR1, 4, 7, 11
1799	AAWYELTPAETTVRLR	NS3	B35	DR1, 4
1827	TAYSQQTRGLLG	NS3	A24	DR1, 7, 11
1426	HMWNFISGIQYLAGLSTLPGNPA	NS4	A2	DR1, 4, 7, 11
1798	IGLGKVLVDILAGYGAGVAGALVAFK	NS4	A2, A3, A11	DR1, 4, 7
1829	SMSYTWTGALITP	NS5	A2, B7	DR1, 7, 11

Characteristics of preferred combination of "hotspots II":
Number of peptides: 7
Potential interactions of peptides via Cysteins: 1 Cys (1846);
potential formation of homodimers of 1846.
Number HCV antigens covered: 5 (core, E2, NS3, NS4, NS5)
HLA class I alleles covered: at least 7
A2 (5 times), A3 (2 times), A11 (2 times), A24 (1 time)
B7 (2 times), B35 (1 time)
Cw7 (1 time)

HLA class II covered: multiple by promiscuous peptides
     DR1 (6 times), DR4 (5 times), DR7 (5 times), DR11 (5 times)

The preferred combination of hotspots II consists of 7 peptides
derived from conserved regions (>80% in genotypes 1, 2, 3) of 5
different HCV antigens. In total at least 7 important HLA class
I alleles are covered (1-5 times), providing a population cover-
age of 81-90% in Europe, 89-91% in U.S. Caucasians and 85-88% in
Japanese people (estimated on numbers from HLA-prevalence stud-
ies in Gjertson and Terasaki, eds., HLA 1998, American Society
of Histocompatibility and Immunogenetics, Lenexa, Kansas, USA).
Similarly, several important HLA class II alleles are covered,
mostly by so called promiscuous peptides (binding to more than
one HLA class II allele). Individual particularly prevalent al-
leles (DR1, 4, 7, 11) are independently covered 5-6 times. Im-
portantly, DR11 is covered 5 times. Presence of this allele has
been linked to spontaneous recovery from HCV infection (Thursz
et al., 1999 Lancet: 354(9196):2119-24).

In contrast to combination I, combination II has a distinct
smaller number of peptides (7 versus 12), which has potential
advantages in manufacturability, formulation and competitive
production of such a product. Importantly, only one peptide with
a cystein side-chain remains (1846). Therefore, only homo-dimers
of 1846, but no heterodimers or oligomers could be formed in
this combination.

At the same time, combination II covers the same 5 HCV antigens
and a similar number of both HLA class I and II alleles as com-
bination I. Hence, the arguments discussed in the previous sec-
tion, hold also true for combination II.

From a formulation development viewpoint, combination II has
several advantages over combination I (only 7 instead of 12 com-
ponents). Most importantly, only one peptide (1846) caries a
free cystein side-chain. Thus, only homodimers od 1846, but no
hetero- or oligomers might be formed.

Example VII: T-cell immunogenicity results from the HCV vaccine-

102 dose optimisation trial

In the course of a clinical trial a number of peptide/poly-arginine mixtures were applied as vaccine to healthy volunteers. Each group consisted of 12 subjects, positive for HLA-A2. Subjects received 4 vaccinations in monthly intervals (at visits 3 to 6). Blood for immunological analyses was drawn at visits 1 to 6, one month after last vaccination (visit 7) and 3 months after last vaccination (visit 8). In order to monitor the clinical trials, Intercell has set-up state-of-the-art T cell assays to determine immunological endpoints under GLP/GCP compliance: Interferon-gamma ELIspot Assay, T cell Proliferation Assay, HLA-tetramer/FACS assay. These assays allow reliable measurements of epitope-specific T cell responses induced by the therapeutic HCV vaccine. The vaccine-induced T cell immune responses serve as surrogate parameters of efficacy (Keilholz et al., J Immunother. 2002 Mar-Apr;25(2):97-138).

As primary endpoint T-cell immunogenicity was determined by a T-cell proliferation assay:
The proliferation assay allows detection of peptide-specific T cells in biological samples like human blood. The basis of the assay is that, T cells upon stimulation with a peptide specifically recognised by their T cell receptor, react by secretion of cytokines and subsequent proliferation. Proliferation of cells can be measured by a variety of means; among the most sensitive approaches ranks incorporation of radioactively labeled thymidine into DNA synthesised prior cell division. This reaction can be carried out in a 96-well plate. Each well contains a fixed number of cells, which are cultured in the presence of antigen/peptide for a couple of days. For the last 16-20 hours thymidine labelled with tritium (3H-thymidine) is added. Cells are then harvested onto a filter plate: medium containing free radioactivity is washed away, whereas DNA sticks to the filter. Incorporated radioactivity can be quantified by means of a beta-scintillation counter. The usual output is given as counts-per-minute (cpm).

As secondary endpoints T-cell immunogenicity was determined by interferon gamma ELIspot

ELIspot allows quantification of peptide-specific, functional
(i.e. cytokine-secreting) T cells in biological samples like hu-
man blood. The basis of the assay is that, T cells upon stimula-
tion with a peptide specifically recognised by the T cell re-
ceptor react by secretion of cytokines like IFN-gamma. This
reaction can be carried out in a 96-well plate. The filter-
wells of this plate are coated with a Mab specific for IFN-
gamma. Consequently, each cell secreting IFN-gamma leaves an
IFN-gamma spot, which can be visualised with a subsequent color
reaction. Spots can be counted using automated plate readers.
Numbers obtained are a measure for the frequency of peptide-spe-
cific, IFN-gamma-secreting T cells in the sample.

**As additional secondary endpoint HLA-tetramer/FACS analysis was
performed.**
HLA class I tetramers, soluble recombinant forms of a complex of
HLA molecule and antigenic peptide, bind the antigen-specific T
cell receptor used for T cell recognition. By using flow cyto-
metry with fluorescent tetramers, antigen-specific CD8+ T lymph-
ocytes can be reliably enumerated and characterised. The assay
uses HLA-A*0201 custom-made iTag™-tetramers produced by Beckman
Coulter Immunomics complexed with HCV vaccine class I epitopes.

Subjects were classified as responders if they showed signific-
ant T-cell responses at any of visits 4 to 8 and had no response
prior treatment.

Maximum Responder Rates (against any peptide, at any of visits 4
to 8, in any of the three T-cell assays of up to 92% versus 25%
in a control group of 25 subjects receiving saline were
achieved.

Responder Rates per peptides Ipep89, Ipep84 and Ipep1426:
As example here responses against T-cell epitope hotspot pep-
tides Ipep89 (class I T-cell epitope), Ipep84 and Ipep1426 are
shown. These responses were obtained after a cycle of 4 monthly
vaccinations. For group B the vaccine contained poly-L-Arginine
only. For Group C the vaccine contained a mixture of 5 peptides,
including Ipep89, Ipep84 and Ipep1426. For Group G the vaccine
contained a mixture of the same 5 peptides as in Group C plus

poly-L-Arginine as a fully synthetic T-cell adjuvant.
Table 19 shows that in control group B no responders in ELIspot
and HLA-tetramer/FACS analysis and 3 responders with in total
only 3 positive visits were seen. The latter can be interpreted
as the rate of false-positive responders obtained in prolifera-
tion assay.
In group C containing only peptides but no poly-L-Arginine, 1
responder against Ipep84 in ELIspot, 5 and 4 responders against
Ipep84 and Ipep1426 respectively in proliferation and 4 respon-
ders against Ipep89 in HLA-tetramer/FACS analysis were seen.
Finally group G (same peptides as in C plus poly-L-arginine)
showed responders in all three assays. Importantly, especially
consistent (all three peptides) and sustained (see number of
total positive visits) ELIspot responses giving an indication
for functional T-cells were dependent on poly-L-arginine as syn-
thetic T-cell adjuvant.

Table 19: Responders per peptide and total number of positive visits in Groups B, C and G

TREATMENT	PEPTIDE	REV/REIV RESPONDERS n/N (%)	TOTAL POSITIVE VISITS n	RPII RESPONDERS n/N (%)	TOTAL POSITIVE VISITS n	RFI RESPONDERS n/N (%)	TOTAL POSITIVE VISITS n	VACCINE RESPONDERS n/N (%)	TOTAL POSITIVE VISITS n
B (poly-Arg only)	Ipep89	0	0	----	--	0	0	0	0
	Ipep84	0	0	3	3	----	--	3	3
	Ipep1426	0	0	0	0	----	--	0	0
C (peptide only)	Ipep89	0	0	----	--	4	4	4	4
	Ipep84	1	1	5	14	----	--	6	15
	Ipep1426	0	0	4	9	----	--	4	9
G (peptide/poly-Arg)	Ipep89	3	5	----	--	5	5	5	8
	Ipep84	3	6	6	11	----	--	8	17
	Ipep1426	5	13	6	11	----	--	7	17

KEY: REV/REIV RESPONDERS=ELISPOT RESPONDERS (CLASS I OR CLASS II);

n/N (%) = percentage of responders per peptide divided by total number of responders in treatment group
RPII RESPONDERS=T-CELL PROLIFERATION RESPONDERS (CLASS II ONLY);
RFI RESPONDERS= HLA-TETRAMER/FACS RESPONDER (CLASS I ONLY);
VACCINE RESPONDER=ANY OF REV/REIV, RPII OR RFI

1. T-CELL PROLIFERATION RESPONSES ONLY CALCULATED ON IPEP 1426.

2. FACS RESPONSES ONLY CALCULATED ON IPEP 89.

Example of an individual course (ELIspot): subject 5 from group G

Table 20 shows the interferon-gamma ELIspot results obtained from serial blood draws of a particular subject from group G (5 peptides+poly-L-arginine). At visit 5, one month after the second vaccination already the two class II epitopes Ipep84, 1426 show responses. After the third vaccination a peak in all responses is reached and also the class I epitope Ipep89 shows a response. Moreover, Ipep1426 shows a sustained response even 3 months after the last vaccination at visit 8.

Table 20: ELIspot values of subject 5 from group G) for Ipep89,
84, 1426 over time
(spots per 1 million PBMC, background subtracted; non-signific-
ant values were set to zero))

	Visit 1	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7	Visit 8
Ipep89	0	0	0	0	22	0	0
Ipep84	0	0	0	18	45	0	0
Ipep1426	0	0	0	22	92	16	16

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